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13. ABSTRACT *(Maximum 200 Words)*

The overall aim of this research is to use a recombinant multiply deleted genomic herpes simplex virus type-1 (HSV-1) based gene transfer vector to test the hypotheses that gene transfer of the glial derived neurotrophic factor (GDNF) or bcl-2 can slow or prevent the apoptotic death of nigral neurons in animal models of PD, and that expression of tyrosine hydroxylase (TH) can improve symptoms.

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INTRODUCTION:

As proposed in the original grant, in order to determine whether the effects of GDNF and Bcl-2 delivered by HSV-mediated gene transfer might be additive, we examined whether delivery of the genes coding for GDNF and bcl-2 using two different recombinant genomic HSV-based vectors together would increase the survival of SN neurons after 6-OHDA administration. The results are detailed in the manuscript which is attached, and are summarized here. [The figures are included with the manuscript in the appendix].

BODY:

Transgene expression in vivo. We previously reported the expression of human bcl-2 RNA in the SN of rats injected with vector THZ/S-bcl2. GDNF expression in vector-injected rat SN was examined by immunocytochemistry using an antibody against human GDNF. Rats injected with DHGD in the SN exhibited numerous GDNF-IR neurons around the injection site, indicating that human GDNF transgene protein was expressed until the time of 6-OHDA lesioning (Figure 2). Control vector-injected rats showed no GDNF immunoreactivity (Figure 2).

THZ/S-bcl2 vector, DHGD vector and coinfection with those two vectors reduce amphetamine-induced ipsilateral rotational behavior. Rats were injected with D-amphetamine (5 ma/kg body weight i.p.) 14 days after 6-OHDA lesioning (21 days after vector or control administration) and their behavior recorded for 90 min. Amphetamine-induced release of DA causes animals with a unilateral lesion of nigrostriatal DA system to turn toward the lesioned striatum. In control lesioned rats injected with either PBS of the lacZ-expressing vector DHZ, ispilateral rotational behavior (approximately 5 turns/min) towards the lesioned hemisphere was observed. Rats injected with THZ/S-bcl2, DHGD, or both vectors together exhibited a significant reduction in ipsilateral behavior compared with control groups (Figure 3).

Both THZ/S-bcl2 and DHGD vectors protect DA neurons from 6-OHDA toxicity. The protection of the nigral DA neurons was evaluated by counting the numbers of TH-IR and FG-labeled cells in the SN bilaterally. In the control animals (PBS- and DHZ-injected), intrastriatal injection of 6-OHDA (5 μl) resulted in the loss of more than 70% of FG-labeled neurons compared to the contralateral unlesioned side. The loss of TH-IR cell bodies (approximately 40%) compared to the uninjected contralateral side was not as great as the loss of FG-labeled neurons. This reflects the fact that while all the terminals of FG-labeled cells were exposed to 6-OHDA which was injected at the same coordinates a week after the FG, TH-IR cells include a population that project to uninjected (i.e. unlesioned) regions of striatum, and were therefore not affected by 6-OHDA. Injection of the THZ/S-bcl2 vector 1 wk prior to lesioning resulted in a 30% increase in the number of surviving FG-labeled cells in lesioned striatum, which represents almost a 2-fold increase in cell survival (Figures 4 and 5). Injection of the GDNF-expressing vector DHGD similarly increased the number of surviving FG-labeled cells by 30% (Figures 4 and 5).

Injection of the bcl-2 expressing vector THZ/S-bcl2 increased the number of surviving TH-IR neurons by 20%, representing a 1/3 increase compared to control (PBS or lacZ-vector injected SN), and injection of the GDNF-expressing vector increased the number of surviving TH-IR neurons by 27%, which represents an increase of 1/2 compared to control animals (Figures 6 and 7). These experiments are in agreement with previous reports using other vectors to deliver and express GDNF and our own results using an HSV vector to deliver and express Bcl-2.

HSV-mediated co-delivery of Bcl-2 and GDNF was more effective than either Bcl-2 or GDNF alone in protecting SN neurons form 6-OHDA toxicity. Injection of both THZ/S-bcl2 and DHGD simultaneously resulted in a 30% increase in the number of surviving FG-labeled cells in the SN compared to animals injected with either the THZ/S-bcl2 alone, or with vector DHGD alone (Figures 4 and 5). Co-inoculation of the THZ/S-bcl2 and DHGD vectors resulted in a an increase (20% compared to bcl-2, 10% compared to GDNF) in the number of surviving TH-IR cells compared to animals injected with either vector alone (Figures 6 and 7). The comparison to bcl-2 was statistically significant, while the comparison to GDNF alone failed to achieve statistical significance.

KEY RESEARCH ACCOMPLISHMENTS:

- Construction of LAP2-HCMV: GDNF vector in a highly defective vector background for evaluation of long-term gene expression in brain.

- Evaluation of recombinant latency promoters driving reporter gene expression have been extensively studied in neuronal cell culture models and in the peripheral nervous system. These studies revealed that the combination of LAP2 with the HCMV promoter produced enhanced long-term levels of transgene product.

- Combined GDNF and Bcl-2 vectors were more effective in inhibiting substantial nigral nerve degeneration in a rat Parkinson's disease model than either gene alone.

REPORTABLE OUTCOMES:

Articles in peer-review journals:

Fink, D.J., DeLuca, N.A., Yamada, M., Wolfe, D.P., and Glorioso, J.C. Design and application of HSV vectors for neuroprotection. Gene Therapy, 7:115-119, 2000.

Liu, T., Khanna, K.M., Chen, X., **Fink, D.J.**, and Hendricks, R.L. CD8+ T cells can block herpes simplex virus type I reactivation from latency in sensory neurons. J. Experimental Med 191:1459-1465, 2000.

Fink, D.J. Gene Therapy for Canavan Disease? Annals Neurology, 48:9-10, 2000

Chen, X., Li, J., Mata, M., Goss, J.R., Glorioso, J.C., and **Fink, D.J**. The ICP0 protein of herpes simplex virus 1 does not accumulate in the nucleus of primary neurons in culture. J. Virology, *in press.*

Goss, J.R., Goins, W.F., Glorioso, J.C., and **Fink, D.J.** Antinociceptive effects of a genomic herpes simplex virus expressing human proenkephalin in rat dorsal root ganglion. *Submitted.*

Yamada, M., Oligino, T., Mata, M., Goss, J.R., Glorioso, J.C., and **Fink, D.J.** Herpes simplex virus vector-mediated expression of Bcl-2 protects spinal motor neurons from degeneration following root avulsion. *Submitted.*

Book Chapters:

Soares, M. K., Goins, W.F., Glorioso, J.C., **Fink, D.J.** Advances in Engineering HSV vectors for gene transfer to the nervous system. Gene Therapy Technologies and Regulations: From Laboratory to Clinic. J. Wiley and Sons Ltd, United Kingdom, pp 127-163. 1999.

Wolfe, D., Goins, W., **Fink, D.,** and Glorioso, J. Design and Use of Herpes Simplex Viral Vectors for Gene Therapy. Gene Therapy: Therapeutic Mechanisms and Strategies. Lasic &Templeton (eds), Marcel Dekker, Inc., New York, NY. pp. 81-108, 2000

Presentations:

The American Neurological Association, Platform presentation of abstract at plenary session (presented by J. Goss). "Antinociceptive effect of gene transfer with an HSV vector coding for human preproenkephalin"
Seattle, WA, October 1999.

4th Gene Delivery and Cellular Protein Expression Conference, "Modifying the structure and function of the nervous system using genomic herpes virus vectors"
Lake Tahoe , CA, October, 1999.

Winter Conference on Brain Research, Workshop Organizer and Presenter, "Treatment of Neurologic Disease with Herpes Vectors
Breckenidge, CO, January, 2000

University of Navarra, "Studies of Gene Therapy for Parkinson's Disease Using Herpes Vectors:
Pamplona, Spain, June, 2000

American Thyroid Association Meeting, Knoll Pharma State of the Art Lecturer "Vector targeting strategies for gene therapy"
Palm Beach,FL, October, 1999

7th Meeting of the European Society of Gene Therapy, "Long-term systemic delivery of nerve growth factor following peripheral infection with a replication defective HSV-1 vector"
Munich, Germany, November 1999

University of North Carolina at Chapel Hill, "Gene therapy using herpesvirus vectors" Chapel Hill, NC, December 1999

Keystone Symposia—Gene Therapy: The Next Millennium, "HSV vectors for treatment of peripheral nervous system disease"
Seattle, WA, January, 2000

Indiana University School of Medicine, Department of Microbiology & Immunology, "Gene therapy of nervous system disease using HSV vectors"
Indianapolis, IN, April, 2000

University of Illinois at Chicago, "Gene therapy of nervous system disease using HSV vectors", Chicago, IL, May, 2000

EUROCANCER 2000, "Herpes-based vectors accommodating multiple genes"
Paris, France, June, 2000

GBK Conference, Keynote Speaker: "Gene therapy: Pioneering the future of molecular medicine"
Dusseldorf, Germany, June, 2000

CONCLUSIONS:

The results of our studies show that the two factors, GDNF and Bcl-2, acting together are more effective than either factor alone in blocking 6-OHDA toxicity, and thus supports the possible utility of a combination therapy in the treatment of Parkinson's disease.

Bcl-2 and GDNF delivered by HSV-mediated gene transfer act additively to protect dopaminergic neurons from 6-OHDA induced degeneration

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Abstract

Previous studies have demonstrated that either the neurotrophin glial derived neurotrophic factor (GDNF) and the anti-apoptotic peptide Bcl-2 delivered into striatum by a viral vector protect dopaminergic neurons of the substantia nigra in vivo from degeneration induced by the administration of the neurotoxin 6-hydroxydopamine (6-OHDA). In this study we used recombinant, replication incompetent, genomic herpes simplex virus (HSV)-based vectors to deliver the genes coding for Bcl-2 and GDNF into rat substantia nigra (SN) one week prior to 6-OHDA injection into the striatum. Vector mediated expression of either Bcl-2 or GDNF alone each resulted in a doubling in cell survival as measured by retrograde labeling with fluorogold (FG), and a 50% increase in tyrosine hydroxylase immunoreactive (TH-IR) neurons in the lesioned SN compared to the unlesioned side. Gene transfer of Bcl-2 and GDNF were equivalent in this effect. Co-administration of the Bcl-2-expressing vector with the GDNF-expressing vector improved the survival of lesioned SN neurons as measured by FG labeling by 33%, and by the expression of TH-IR by 15%. These results suggest that the two factors delivered together act in an additive fashion to improve DA cell survival in the face of 6-OHDA toxicity.

Intrastriatal injection of 6-OHDA to cause progressive loss of the dopaminergic terminals of SN neurons (37, 38) is widely used to model the degeneration of neurons of the SN in Parkinson's disease (PD). Several different treatments are effective in protecting these neurons from toxin-induced degeneration. Glial cell line-derived neurotrophic factor (GDNF) was originally identified by its ability to support the survival of dopaminergic neurons of the SN in vitro (26). It is a member of a family of related neurotrophic factors in the transforming growth factor-β (TGF-β) family of basic, dimeric secretory proteins with a cysteine knot structure (2). The receptor complex consists of a GDNF-specific binding component, the GDNF-family receptor α1 (GFRα-1) (42) and a transmembrane Ret receptor tyrosine kinase (16, 43) that triggers several intracellular signaling pathways including the Ras-MAPK (48), phosphoinositol-3-kinase (PI3K) (45), Jun N-terminal kinase (JNK) (45) and PLCγ (7) dependent pathways. Recombinant GDNF administered by direct injection or continuous infusion protects DA neurons of the SN from 6-OHDA toxicity (4, 20, 22, 39, 41). Alternatively, transfer of the gene coding for GDNF using a recombinant adenovirus- (5, 6, 11, 12), or adenoassociated virus- (23, 28, 29) based vectors, and implantation of encapsulated GDNF-producing cells (27) have been demonstrated to protect DA neurons from degeneration following 6-OHDA administration. The specific mechanisms involved in the protection of SN neurons against 6-OHDA toxicity have not been defined, but may involve protection from both apoptosis and necrosis.

Evidence suggests that death of DA cells caused by 6-OHDA proceeds at least in part through pathways with features of apoptosis. 6-OHDA administration results in cell death with morphologic characteristics of apoptosis in vivo (30) and in PC12 cells in vitro (46, 47). Dopamine-induced apoptosis of PC12 cells can be inhibited by overexpression of the anti-apoptotic peptide Bcl-2 (33), and primary cultures of SN neurons derived from transgenic mice that overexpress Bcl-2 are resistant to 6-OHDA toxicity in vitro (32). Using a recombinant genomic HSV vector we recently demonstrated that delivery and expression of the gene coding for bcl-2 protects dopaminergic neurons of the SN from death induced by 6-OHDA administration, and at the same time preserved the neurotransmitter phenotype of the lesioned cells (50). These results are similar to those reported in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

model of PD, in which transgenic mice overexpressing bcl-2 were demonstrated to be resistant to MPTP toxicity (32, 51).

Bcl-2 is one member of a large family of proteins homologous to the nematode protein CED-9 that plays a critical role in inhibiting apoptosis in the face of a wide variety of cytotoxic insults (1), It forms a heterodimer with pro-apoptotic members of the same family, acting to prevent mitochondrial membrane permeabilization and consequent release of cytochrome c from mitochondria (25) thus averting the subsequent activation of the effector caspases that cleave a variety of intracellular peptides including major structural elements of the cytoplasm and nucleus that result in the stereotypic morphologic and biochemical changes that characterize apoptotic cell death (17).

In order to determine whether the effects of GDNF and Bcl-2 may be additive, we examined whether delivery of the genes coding for GDNF and bcl-2 using two different recombinant genomic HSV-based vectors together would increase the survival of SN neurons after 6-OHDA administration.

Materials and methods

Viral constructs. The THZ/S-Bcl2 vector was constructed as previously described (50). The DHGD vector was constructed by first cotransfecting an ICP4$^-$, ICP27$^-$ virus with the p41ICP0Lac7 plasmid as previously described (24). This plasmid contains the E. coli *lacZ* gene flanked by *Pac*I sites and U_L41 sequences. Recombinant viruses containing the *lacZ* gene in the U_L41 locus were selected by X-gal staining; the LacZ reporter was then removed by *Pac*I digestion. The digested viral DNA was cotransfected with a plasmid containing the human GDNF gene construct flanked by U_L41 sequences. Recombinants containing the GDNF transgene in place of the *lacZ* reporter gene were identified from among clear plaque producing viruses by Southern blot analysis. A schematic illustration of the viral constructs is shown in Figure 1.

Animals. Female Sprague-Dawley rats (250 - 300 g) were randomly divided to receive an injection of either 4 µl of phosphate buffered saline (PBS, n=7), 4 µl of DHZ (n=8), 4 µl of THZ/S-bcl2 (n=7) or 4 µl

of DHGD (n=7), or a co-infection of 4 µl of THZ/S-bcl2 and 4 µl of DHGD (n=6), 7 days before 6-OHDA lesioning.

Surgical procedures. Surgeries were performed according to the protocol of Sauer and Oertel (38). Under chloral hydrate anesthesia (400 mg/kg i.p.), rats received bilateral striatal injections (coordinates anterior-posterior +1.0, medial-lateral 3.0, dorsal-ventral -5.0 relative to the bregma using the atlas of Paxinos and Watson (34)) of fluorogold (3 µl of 2% FG in 0.9 % saline; Fluorochrome, Denver, CO) to retrogradely label a subpopulation of dopaminergic neurons within the SN that project to the site of the lesion. At the same time, rats were injected unilaterally into the region of the substantia nigra (SN) (AP -4.0, ML -2.0, DV -8.0) with either THZ/S-bcl2 and/or DHGD vectors or the DHZ control vector at a rate of 1 µl/min. Seven days after FG labeling and vector inoculation the rats were reanesthetized and injected with 6-OHDA (20 µg of free base, 4 µg/µl in 0.02% ascorbic acid) unilaterally into striatum using the same coordinates as the FG injection, ipsilateral to the vector injection into SN. Fourteen days after 6-OHDA lesioning, the rats were injected with D-amphetamine (see below) to examine amphetamine-induced rotational behavior. Two hours following amphetamine injection, the rats were deeply anesthetized with an excess dose of chloral hydrate and perfused through the heart with PBS followed by 300 ml of [illegible] buffered formalin phosphate. The brains were removed and post-fixed with 10% buffered formalin phosphate overnight, cryoprotected with 30% sucrose in PBS for 3 days, and 40-µm sections mounted directly for examination of FG-labeled cells or processed for tyrosine hydroxylase (TH) immunocytochemistry.

Behavioral testing. Rats were injected with D-amphetamine sulfate (5 mg/kg body weight i.p., Sigma, St. Louis, MO) and placed in a rotometer (Coulbourn Instrument) where the number of clockwise and counter-clockwise turns was counted for 90 min. The rotation score is expressed as the number of net rotations per minute in the lesioned (ipsilateral) direction.

Immumocytochemistry and cell counting. Floating sections were preincubated with 5% normal goat serum (NGS), incubated with a rabbit anti-TH antibody (1:500, Chemicon) overnight at room temperature, followed by a secondary antibody conjugated to biotin (1:200, Vector Laboratories) for 2 hr and detected

with diaminobenzidine by using a commercial kit (Vectastain ABC kit; Vector laboratories). The number of surviving FG-labeled and TH-immunoreactive cells through the SN (four to seven sections per animal for each technique) was counted by an observer blinded to the treatment group. The number of surviving (FG-positive) or TH-immunoreactive cells was expressed as a percentage of the similar cells counted on the intact (unlesioned, contralateral) side. The statistical significance of the difference was determined by ANOVA (StatView, SAS Institute), using Bonferroni's correction for the multiple comparisons employed.

Transgene expression. Transgene expression of GDNF was examined by immunocytochemistry. 10 μm frozen sections of the SN obtained from rats injected 7 days prior to sacrifice with 4 μl of DHGD were mounted on gelatin-coated slides. After fixing for 15 min with 4 % paraformaldehyde in 0.1 M phosphate buffer, sections were preincubated with 5 % normal horse serum (NHS), incubated overnight at room temperature with goat anti-GDNF antibody (2 μg/ml, R&D Systems), followed by a secondary antibody conjugated to biotin (1:500, Vector Laboratories) for 2 hr and detected with diaminobenzidine by using a commercial kit (Vectastain ABC kit; Vector laboratories).

Results

Transgene expression in vivo. We have previously reported the expression of human bcl-2 in the SN of rats injected with vector THZ/S-bcl2 (50). GDNF expression in vector-injected rat SN was examined by immunocytochemistry using an antibody against human GDNF. Rats injected with DHGD in the SN exhibited numerous GDNF-IR neurons around the injection site, indicating that the GDNF transgene protein was expressed until the time of 6-OHDA lesioning (Figure 2). Control vector-injected rats showed no GDNF immunoreactivity (Figure 2).

THZ/S-bcl2 vector, DHGD vector and coinfection with those two vectors reduce amphetamine-induced ipsilateral rotational behavior. Rats were injected with D-amphetamine (5 ma/kg body weight i.p.) 14 days after 6-OHDA lesioning (21 days after vector or control administration) and their behavior recorded for 90 min. Amphetamine-induced release of DA causes animals with a unilateral lesion of nigrostriatal DA system to turn toward the lesioned striatum. In control lesioned rats injected with either PBS of the lacZ-expressing vector DHZ, ispilateral rotational behavior (approximately 5 turns/min)

towards the lesioned hemisphere was observed. Rats injected with THZ/S-bcl2, DHGD, or both vectors together exhibited a significant reduction in ipsilateral behavior compared with control groups (Figure 3).

Both THZ/S-bcl2 and DHGD vectors protect DA neurons from 6-OHDA toxicity. The protection of the nigral DA neurons was evaluated by counting the numbers of TH-IR and FG-labeled cells in the SN bilaterally. In the control animals (PBS- and DHZ-injected), intrastriatal injection of 6-OHDA (5 μl) resulted in the loss of more than 70% of FG-labeled neurons compared to the contralateral unlesioned side. The loss of TH-IR cell bodies (approximately 40%) compared to the uninjected contralateral side was not as great as the loss of FG-labeled neurons. This reflects the fact that while all the terminals of FG-labeled cells were exposed to 6-OHDA which was injected at the same coordinates a week after the FG, TH-IR cells include a population that project to uninjected (i.e. unlesioned) regions of striatum, and were therefore not affected by 6-OHDA. Injection of the THZ/S-bcl2 vector 1 wk prior to lesioning resulted in a 30% increase in the number of surviving FG-labeled cells in lesioned striatum, which represents almost a 2-fold increase in cell suvival (Figures 4 aned 5). Injection of the GDNF-expressing vector DHGD similarly increased the number of surviving FG-labeled cells by 30% (Figures 4 and 5).

Injection of the bcl-2 expressing vector THZ/S-bcl2 increased the number of surviving TH-IR neurons by 20%, representing a 1/3 increase compared to control (PBS or lacZ-vector injected SN), and injection of the GDNF-expressing vector increased the number of surviving TH-IR neurons by 27%, which represents an increase of 1/2 compared to control animals (Figures 6 and 7). These experiments are in agreement with previous reports using other vectors to deliver and express GDNF (5, 11, 23, 29) and our own results using an HSV vector to deliver and express Bcl-2 (50).

HSV-mediated co-delivery of Bcl-2 and GDNF was more effective than either Bcl-2 or GDNF alone in protecting SN neurons form 6-OHDA toxicity. Injection of both THZ/S-bcl2 and DHGD simultaneously resulted in a 30% increase in the number of surviving FG-labeled cells in the SN compared to animals injected with either the THZ/S-bcl2 alone, or with vector DHGD alone (Figures 4 and 5). Co-inoculation of the THZ/S-bcl2 and DHGD vectors resulted in a an increase (20% compared to bcl-2, 10% compared to GDNF) in the number of surviving TH-IR cells compared to animals injected with either

vector alone (Figures 6 and 7). The comparison to bcl-2 was statistically significant, while the comparison to GDNF alone failed to achieve statistical significance.

Discussion

The principal finding of the experiments reported in this communication is that GDNF and bcl-2 appear to act synergistically in protecting DA neurons of the SN from the neurotoxic effects of 6-OHDA. While HSV vectors expressing either bcl-2 or GDNF alone were effective in protecting those cells from 6-OHDA toxicity, both the survival of DA neurons as measured by the number of retrogradely labeled FG-positive cells and the number of TH-immunoreactive neurons, was greater in animals injected with both vectors simultaneously compared to either vector alone. The additive effect appears more robust when measured by survival of FG-labeled neurons. In part, this reflects the greater selectivity of FG-labeling for neurons that are exposed to the neurotoxin, which also accounts for the survival of only 20% of the FG-labeled neurons, in contrast to the survival of 60% (compared to the unlesioned contralateral side)

GDNF was initially identified as a factor secreted from a glioma cell line that was capable of supporting embryonic ventral midbrain neurons survival in culture (26), although it is not required for survival of these neurons during development (18). GDNF produces its effects through activation of Ret, a receptor tyrosine kinase (2, 9). The Ras-MAPK pathway activated by Ret appears to be necessary for the survival and neurite growth-stimulating actions of GDNF and related members of the GDNF family (15, 44, 49). Signaling through the phosphatidylinositol 3-kinase pathway is required for the differentiation of cultured mesencephalic DA neurons in vitro (35), and the same pathway has been implicated in the formation of lamellopodia in other systems (44, 45), a function that may be related to the extension of neurites. GDNF has also been reported to activate the c-Jun N-terminal kinase (JNK) pathway through phosphorylation of Rho/Rac-related small GTPases (10). GDNF binding to the GDNF receptor may also activate a cytoplasmic Src family tyrosine kinase resulting in phosphorylation of mitogen-activated protein kinase independent of Ret-mediated signaling pathways (36).

6-OHDA exerts its neurotoxic effects on catecholaminergic neurons through the generation of hydrogen peroxide, superoxide, and cytotoxic hydroxyl radicals which directly cause damage to the cells (14). Other

effects including alterations of mitochondrial calcium homeostasis (19) and direct inhibition of mitochondrial complexes I and IV (21) have been described. Several independent lines of indirect support the hypothesis that 6-OHDA-induced death of DA neurons proceeds through apoptotic pathways, and we previously demonstrated that expression of bcl-2 in SN from an HSV vector protected SN neurons from 6-OHDA neurotoxicity in vivo (50). GDNF also has anti-apoptotic effects, and has been shown to inhibit apoptotic cell death of DA neurons of the SN in culture (8, 13).

There are many paths through which apoptosis may be activated (17), and Bcl-2 plays a critical role in many of these pathways by directly or indirectly preventing the release of cytochrome c from mitochondria, thereby inhibiting activation of procaspase-9 and the effector caspase cascade leading ultimately to the proteolytic cleavage of essential structural and functional cellular proteins and resulting in cell death (1, 17). Bcl-2 resides on several intracellular membranes including the cytoplasmic face of the mitochondrial outer membrane, the endoplasmic reticulum, and the nuclear envelope, so that in order to be effective expression must be achieved in the affected cell. Unlike other neurotrophic factors such as nerve growth factor (NGF) or ciliary neuronotrophic factor CNTF (3) which have been clearly demonstrated to function in part through the block of apoptotic pathways, a direct intersection of GDNF activated pathways and the caspase cascade has not previously been demonstrated.

How might the pathways activated by overexpression of GDNF and the apoptotic pathways blocked by overexpression of bcl-2 overlap? One possibility is the independent activation of downstream effectors through alternate pathways. In another model for instance, GDNF has been shown to prevent ethanol induced apoptosis in a GDNF-responsive neuroblastoma cell line; this effect correlates with a block of ethanol induced phosphorylation of JNK, but does not block phosphorylation of the extracellular signal-regulated kinases (ERKs) that are known to effect cell survival (31). Another possibility is that GDNF may directly increase expression of antiapoptotic peptides or otherwise modulate upstream effectors in the caspase cascade. In mesencephalic neurons in culture it has recently been demonstrated that GDNF provides neuroprotection against both bleomycin (BLM) and L-buthionine-[S,R]-sulfoximine (BSO) induced apoptosis; an effect that could be blocked by caspse-3 inhibition (40). In that system, GDNF upregulated expression of bcl-2, and the anti-apoptotic effect of GDNF was prevented by inhibition of

RNA or protein synthesis. GDNF also phosphorylated Akt, but that effect was temporally unrelated to the antiapoptotic effect in the model system employed (40).

Further studies will be required to define the mechanisms through which GDNF and bcl-2 act together to prevent 6-OHDA neurotoxicity to DA neurons. However, the results reported show that the two factors acting together are more effective than either factor alone in blocking that toxicity, and thus supports the possible utility of a combination therapy in the treatment of Parkinson's disease.

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Figure Legends

Figure 1. Viral constructs. Schematic representation of THZ/S-bcl2 and DHGD.

Figure 2. GDNF transgene expression in SN. Seven days after injection of 4 μl of vector DHGD into the SN, GDNF-immunoreactivity could be detected in neurons of the SN (left). A control injected with a similar vector carrying only the E coli β galactosidase (*lacZ*) transgene showed no immunoreactivity.

Figure 3. Amphetamine-induced rotation. Fourteen days after unilateral striatal injection of 6-OHDA behavior was examined after injection of D-amphetamine. Rats that had been injected with THZ/S-bcl2 (bcl2) or DHGD (GDNF) and those receiving co-injection of those vectors (bcl2 + GDNF) displayed a significant reduction in induced ipsilateral rotation compared with PBS-injected or control vector (DHZ)-injected rats. Unlesioned animals showed no clear rotation (-0.5 $\pm$ 0.7 turns/min, data not shown). *$p<0.01$ compared with bcl2, GDNF or bcl2 + GDNF; $^{+}p<0.05$ compared with bcl2, GDNF or bcl2 + GDNF. All values are means $\pm$ SEM.

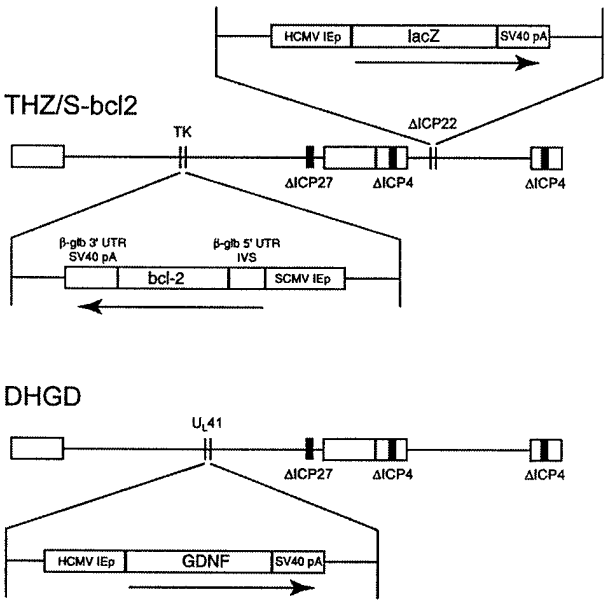
Figure 4. FG-labeled cells in lesioned/vector-injected and intact SN 14 days after 6-OHDA injection. Representative photomicrographs from animals injected into SN with DHZ (control), THZ/S-[illegible] DHGD (GDNF) and coinjected with THZ/S-bcl2+DHGD (bcl2 + GDNF). Right (lesioned[illegible] with 6-OHDA into striatum and vector into SN. Left (intact) show contralateral unlesior[illegible] sections from the same animal.

Figure 5. Counts of FG-labeled neurons in lesioned SN 14 days after 6-OHDA injection. *$p<0.005$ compared with bcl2, GDNF and bcl2+GDNF. $^{+}p<0.01$ compared with bcl2, GDNF and bcl2+GDNF; #$p<0.01$ compared with bcl2 and GDNF. All values are means $\pm$ SEM.

Figure 6. TH-IR cells in lesioned/vector-injected and intact SN 14 days after 6-OHDA injection. Representative photomicrographs from animals injected into SN with DHZ (control), THZ/S-bcl2 (bcl-2), DHGD (GDNF) and coinjected with THZ/S-bcl2+DHGD (bcl2 + GDNF). Right (lesioned) were injected

with 6-OHDA into striatum and vector into SN. Left (intact) show contralateral unlesioned/uninjected sections from the same animal.

Figure 7. Survival of TH-IR DA neurons in SN 21 days after the vector injection. *$p<0.05$ compared with bcl2, GDNF and bcl2+GDNF. $^{+}p<0.05$ compared with bcl2, GDNF and bcl2+GDNF; #$p<0.05$ compared with bcl2. All values are means $\pm$ SEM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lacZ
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TK
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SV40 pA
β-glb 5' UTR
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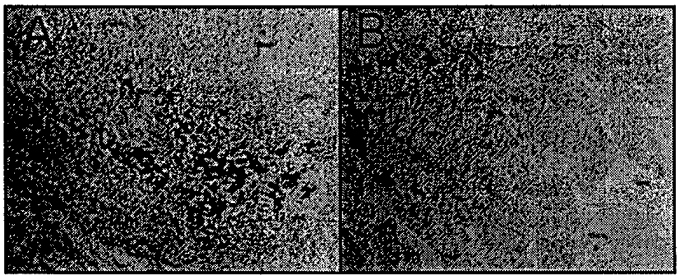
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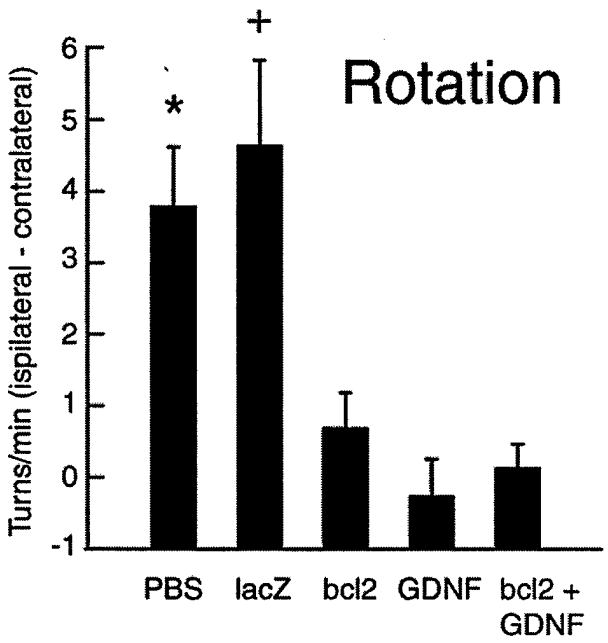
Rotation
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-1
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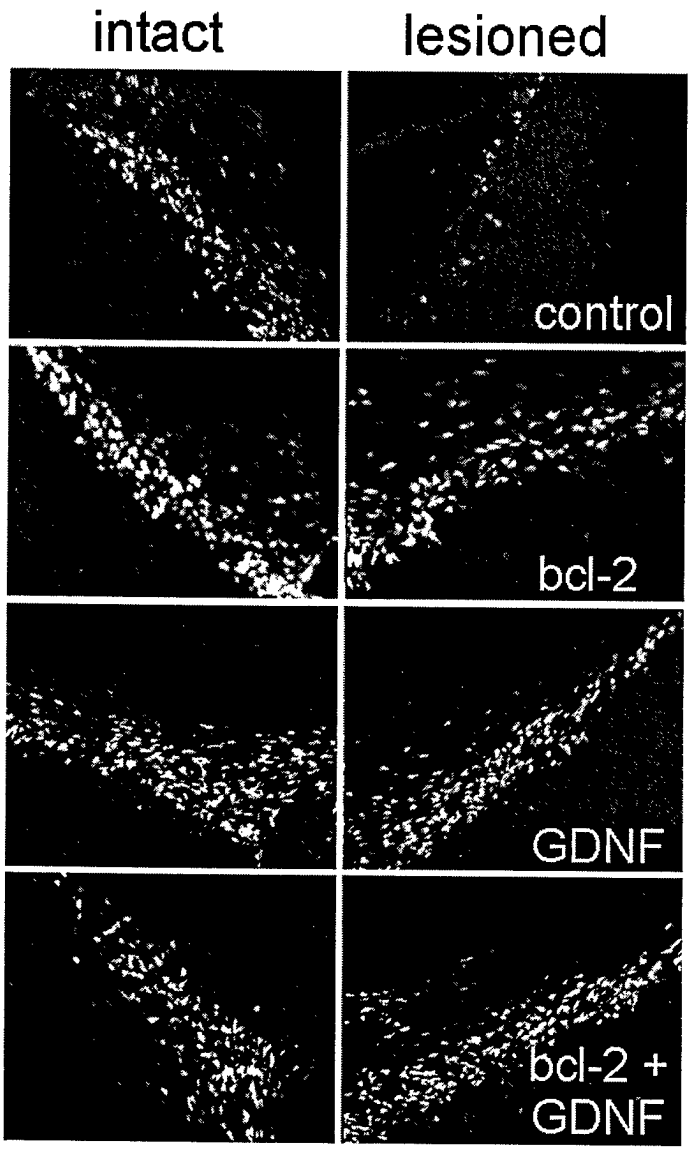
+
PBS
lacZ
bcl2
GDNF
bcl2 + GDNF


intact
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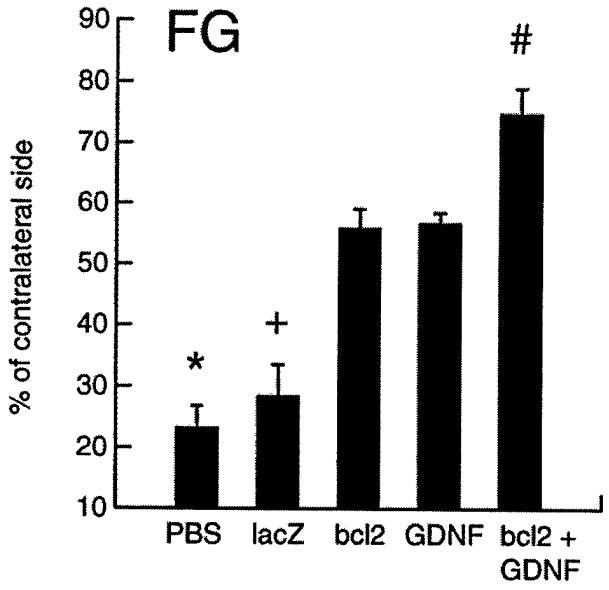
bcl-2
GDNF
bcl-2 +
GDNF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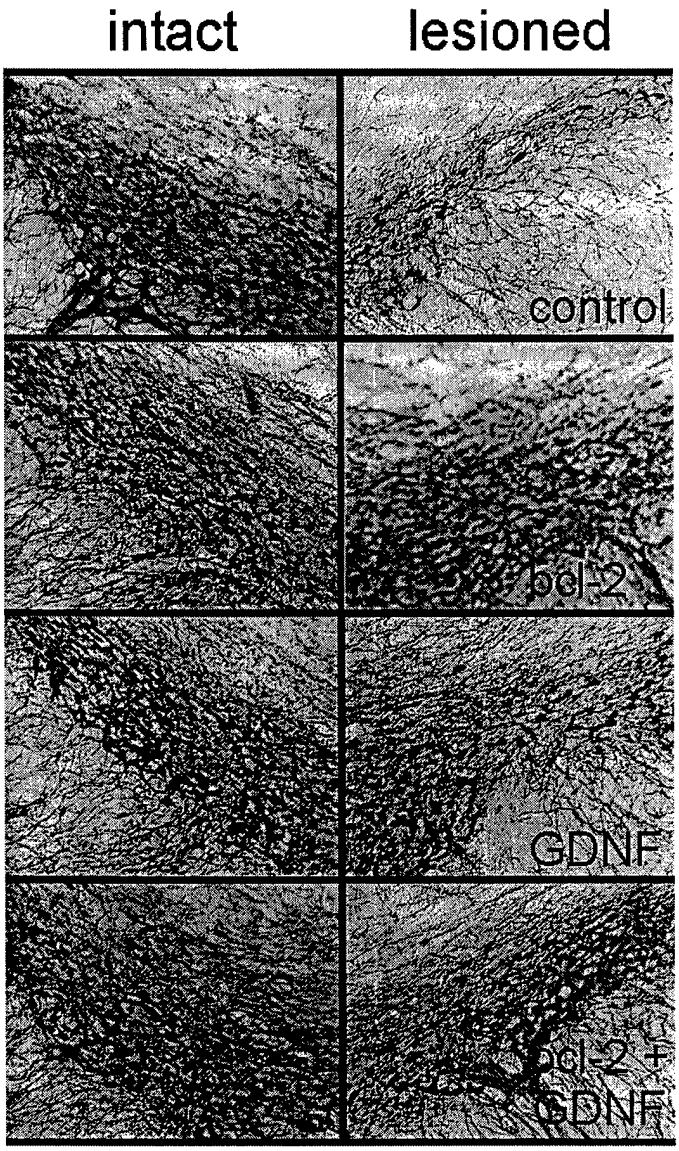
bcl2 + GDNF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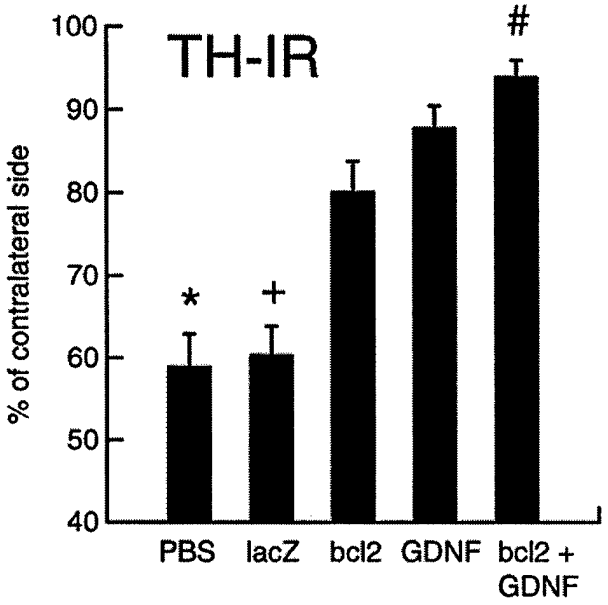
TH-IR
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bcl2 + GDNF